(19)日本国特許庁（ＪＰ）　(12)**公開特許公報（Ａ）**　(11)特許出願公開番号
特開2002－241385
（P2002－241385A）
(43)公開日　平成14年８月28日(2002.8.28)

| (51)Int.Cl.[7] | 識別記号 | ＦＩ | | テーマコード（参考） |
|---|---|---|---|---|
| Ｃ０７Ｆ　9/10 | | Ｃ０７Ｆ　9/10 | Ｚ | ４Ｈ０５０ |
| | | | Ａ | |
| | | | Ｂ | |

審査請求　未請求　請求項の数４　ＯＬ　（全８頁）

(21)出願番号　特願2001－38430(P2001－38430)

(22)出願日　平成13年２月15日(2001.2.15)

(71)出願人　000006884
株式会社ヤクルト本社
東京都港区東新橋１丁目１番19号
(72)発明者　酒井　正士
東京都港区東新橋１丁目１番19号　株式会社ヤクルト本社内
(72)発明者　海老名　里夏
東京都港区東新橋１丁目１番19号　株式会社ヤクルト本社内
(74)代理人　100092082
弁理士　佐藤　正年　（外１名）

最終頁に続く

(54)【発明の名称】　ホスファチジルセリンの分画法

(57)【要約】
【課題】　天然あるいは人工的に調製されたリン脂質混合物からホスファチジルセリンを簡便に濃縮する分画法を得る。
【解決手段】　ホスファチジルセリンを含むリン脂質混合物をアルコール類に溶解した後、該溶解液中に金属塩を添加することによりホスファチジルセリンを不溶化せしめ、該不溶部を分離するもの。

1

【特許請求の範囲】

【請求項１】　ホスファチジルセリンを含むリン脂質混合物をアルコール類に溶解した後、該溶解液中に金属塩を添加することによりホスファチジルセリンを不溶化せしめ、該不溶部を分離することを特徴とするホスファチジルセリンの分画法。

【請求項２】　前記金属塩として、リチウム塩、カリウム塩及びナトリウム塩から選ばれる１種又は２種以上を用いることを特徴とする請求項１に記載のホスファチジルセリンの分画法。

【請求項３】　前記金属塩として、塩化リチウム、塩化カリウム又は塩化ナトリウムを用いることを特徴とする請求項１又は２に記載のホスファチジルセリンの分画法。

【請求項４】　前記アルコール類として、エチルアルコールを用いることを特徴とする請求項１～３の何れかに記載のホスファチジルセリンの分画法。

【発明の詳細な説明】

【０００１】

【発明の属する技術分野】本発明は、リン脂質混合物からホスファチジルセリンを濃縮するための分画法に関するものである。

【０００２】

【従来の技術】ホスファチジルセリン（以下、「ＰＳ」と記載する。）は、痴呆症の予防や治療などを目的とした脳機能改善剤の他、免疫性疾患の治療剤や界面活性剤としての利用が期待されている。このＰＳは動物の脳や筋肉に含まれる他、化学合成法やホスホリパーゼＤを使用したホスファチジル基転移反応により人工的に製造することも可能である。

【０００３】ＰＳは主に医薬や食品、化粧品として使用されていることから、前記天然物や反応物等からＰＳを分画し、ＰＳ含量を高めることが重要である。ところが、動物の脳や筋肉に含まれるＰＳ量は少なく、また化学合成法やホスファチジル基転移反応により製造した場合にも、ＰＳ含量の多い製品を安価に製造することは難しいことから、使用に際してはＰＳの濃縮（精製）が必要となる場合が多い。

【０００４】ＰＳの濃縮（精製）法としては、従来、溶媒による分画やカラムクロマトグラフィーが用いられてきた。しかし、溶媒分画のみでは充分なＰＳ純度を得ることは困難であり、一方、クロマトグラフィーのような煩雑な操作は、コスト面、作業性の面で問題があった。このため、ＰＳを安価かつ簡便に濃縮する方法の開発が望まれている。

【０００５】特に、ＰＳは他のリン脂質、すなわち、ホスファチジルコリン（ＰＣ）、ホスファチジルエタノールアミン（ＰＥ）、ホスファチジルイノシトール（ＰＩ）やホスファチジン酸（ＰＡ）等と分離することが困難であるため、これらを含むリン脂質混合物から、ＰＳを安価に濃縮する方法を確立することが望まれている。

2

【０００６】

【発明が解決しようとする課題】本発明者らは上記課題を解決するために鋭意研究を重ねた結果、ＰＳを含むリン脂質混合物をアルコール類に溶解し、さらに金属塩あるいはその溶液を添加することによりＰＳを沈殿せしめ、沈殿部に濃縮できることを見出した。

【０００７】本発明は、天然あるいは人工的に調製されたリン脂質混合物からＰＳを簡便に濃縮する分画法を得ることを目的とする。

【０００８】

【課題を解決するための手段】請求項１に記載された発明に係るＰＳの分画法は、ＰＳを含むリン脂質混合物をアルコール類に溶解した後、該溶解液中に金属塩を添加することによりＰＳを不溶化せしめ、該不溶部を分離するものである。

【０００９】請求項２に記載された発明に係るＰＳの分画法は、請求項１に記載の金属塩として、リチウム塩、カリウム塩及びナトリウム塩から選ばれる１種又は２種以上を用いるものである。

【００１０】請求項３に記載された発明に係るＰＳの分画法は、請求項１又は２に記載の金属塩として、塩化リチウム、塩化カリウム又は塩化ナトリウムを用いるものである。

【００１１】請求項４に記載された発明に係るＰＳの分画法は、請求項１～３の何れかに記載のアルコール類として、エチルアルコールを用いるものである。

【００１２】

【発明の実施の形態】本発明においては、ＰＳを含むリン脂質混合物をアルコール類に溶解した後、該溶解液中に金属塩を添加するという簡略な操作によって、ＰＳを不溶化（沈殿、凝集等）させることにより、不溶物（沈殿部、凝集部等）を分離・濃縮する。これにより、天然あるいは人工的に調製されたリン脂質混合物からＰＳを簡便に濃縮することができる。

【００１３】本発明に用いられるＰＳを含むリン脂質混合物としては、天然物、天然物からの抽出物又は該抽出物を精製したもの、或いは合成リン脂質等ＰＳを含む混合物であれば、いずれを用いてもよい。具体的には、大豆レシチン、菜種レシチン、卵黄レシチン、トウモロコシレシチン或いは綿実レシチンや、化学合成法やホスファチジル基転移反応により調製したリン脂質混合物、牛脳の溶媒抽出物等が挙げられる。中でも、ホスファチジル基転移反応により調製したＰＳを含むリン脂質混合物を用いれば、金属塩を添加した場合の濃縮効果が高く、原料の確保のしやすさやコスト面からも好ましい。

【００１４】また、本発明に用いられる金属塩としては、リチウム塩、ナトリウム塩、カリウム塩、カルシウム塩、マグネシウム塩等の金属塩、あるいはこれらを豊富に含む天然物、例えば、食塩、苦汁、かん水、ドロマ

イト、食用真珠層粉等いずれを用いても良いが、リチウム塩、ナトリウム塩またはカリウム塩を用いることが濃縮効率の点から好ましく、特に塩化リチウム、塩化ナトリウムまたは塩化カリウムが好ましい。これらの金属塩は、１種または２種以上を組み合わせて用いることができる。

【００１５】これら金属塩の添加量は、ＰＳを沈殿させ得る量であれば特に限定されないが、リン脂質１ｇあたり、０．１５～１０ミリモル、特に０．５～５ミリモルであることが、ＰＳの回収率および沈殿中のＰＳ含量が高い点から好ましい。

【００１６】また、本発明に用いられるアルコール類としては、リン脂質混合物を溶解可能なアルコール類であればいずれも好適に用いられるが、中でもメチルアルコール、エチルアルコール、ブチルアルコール、プロピルアルコール、イソプロピルアルコール等の低級アルコール類が好ましい。また、これらの混合物を利用することもできるが、エチルアルコールは食品へ利用し易く、安全面での問題も少ないため、これを用いることが特に好ましい。

【００１７】リン脂質混合物をアルコール類に溶解する際の濃度は特に限定されないが、この混合物が完全に溶解できる以上の量とすることが好ましく、アルコール類の重量に対し１～５０％、特に２～２０％とすることが、ＰＳ濃縮効率や操作性の点から好ましい。

【００１８】本発明において、リン脂質混合物からのＰＳの分画は、例えば以下のようにして行うことができる。まず、ホスファチジル基転移反応法等により調製され、ＰＣ、ＰＥ又はＰＡ等ＰＳ以外のリン脂質を成分中に含むリン脂質混合物を、エチルアルコール等のアルコール類に溶解する。この時、溶解温度等の溶解の条件は特に限定されず、混合物の成分の種類、それらの量等に合わせ好適な条件を選択し用いればよい。

【００１９】こうして得られる溶液中では、ＰＳやＰＣ、ＰＡ等のリン脂質は溶媒層に抽出されるが、場合によっては一部の不溶性成分が生成する。このため、遠心分離、ろ過等の手段により溶媒から不溶性成分（沈殿物、凝集物等）を除いてから金属塩の添加を行う。不溶性成分中にも少量のＰＳが残存している場合には、前記アルコール類による抽出処理は、数度繰り返し行ってもよい。

【００２０】次いで、アルコール溶液に対して、金属塩を添加し、溶媒層に抽出されたＰＳを分画する。すなわち、溶媒層中のＰＳ以外のリン脂質の大部分は、金属塩の添加によっても沈殿しないが、ＰＳはその大部分が沈殿するため、これを回収することによりＰＳの濃縮を行うことができる。このとき、金属塩は粉末のまま加えても、水やアルコール等の溶媒に溶かしてから加えてもよい。その際の各種条件も特に限定されず、混合物の成分の種類、それらの量等に合わせ好適な条件を選択すればよい。具体的には１０℃～３０℃で３０分以上保持してＰＳを不溶化させればよい。

【００２１】金属塩の添加により不溶化されたＰＳは、遠心分離、ろ過、静置分離等の手段により、回収することができる。また、公知の精製手段、例えばカラムクロマトグラフィー等の手段により、更に精製することも可能である。本発明のＰＳ濃縮物は他のリン脂質等の含量が顕著に低下しているため、このような精製手段も比較的簡便に行うことができる。

【００２２】本発明のＰＳ濃縮物は、医薬品、食品、化粧品等の形態で投与することができる。例えばリン脂質の生理効果を訴求する医薬品や栄養補助食品等の形態で用いる場合であれば、カプセル剤、顆粒剤、錠剤、散剤等の固形製剤、或いはシロップ剤等の液状製剤として経口投与することができる。また、経口投与剤でなくとも、注射剤、皮膚外用剤、直腸投与剤等非経口形態で投与することも可能である。

【００２３】各製剤の製造時には、乳糖、澱粉、結晶セルロース、乳酸カルシウム、メタケイ酸アルミン酸マグネシウム、無水ケイ酸等の賦形剤、白糖、ヒドロキシプロピルセルロース、ポリビニルピロリドン等の結合剤、カルボキシメチルセルロース、カルボキシメチルセルロースカルシウム等の崩壊剤、ステアリン酸マグネシウム、タルク、モノグリセリド、蔗糖脂肪酸エステル等の滑沢剤や、その他、医薬・食品として許容され得る成分を適宜使用すればよい。

【００２４】また、同様の生理効果を期待して一般食品形態（「明らか食品」の形態）で用いる場合には、本発明の方法により得られたＰＳ濃縮物をそのまま或いは適宜精製処理したものを油脂、錠菓、発酵乳、飴、調味料、ふりかけ等の飲食品に添加し、常法を用いて製造すればよい。

【００２５】これら医薬品、食品等の形態での使用に際しては、本発明の方法により得られたＰＳが濃縮されたリン脂質組成物を適宜配合することができる。また、ＰＳの生理効果を訴求する場合であれば、その効果を得られかつ過剰摂取等の問題が生じない程度の量、５０ｍｇ～１０００ｍｇ／日程度の摂取が見込まれる量を適宜配合しておけばよい。

【００２６】更に、本発明のリン脂質は乳化剤として用いてもよく、その際には、医薬品、食品、化粧品等へ０．０１～１０％添加するのが好ましい。

【００２７】

【実施例】以下に、実施例を挙げて本発明を説明するが、本発明はこれらに限定されるものではない。

【００２８】実施例１

大豆レシチン（ＰＣ８０：クロクラーン社製）１０．０ｇと大豆油２．０ｇを１００ｃｃメジウム瓶に取り、ここに９０．０ｇ（１００ｍＬ）の酢酸エチルを加えてスターラーで撹拌しながら加温溶解した。Ｌ－セリン１．

２ｇとＰＬＤ－Ｙ１（（株）ヤクルト本社製）１，５００単位を秤り取り、０．１Ｍリン酸ナトリウム緩衝液（ｐＨ７．０）５．８ｍＬを加えて溶解した。

【００２９】ＰＣ８０溶液全量の入ったメジウム瓶を５０℃に保温しておき、ここに５０℃に保温した（Ｌ－セリン＋ＰＬＤ－Ｙ１）溶液の全量を加えて反応を開始し、スターラーで緩やかに撹拌しながら５０℃で５時間反応させた。３０分間氷冷してリン脂質を沈殿させて回収した後、熱湯中に２０分放置して酵素を失活させた。

【００３０】回収したリン脂質層にエタノール４０ｍＬを加えて良く混合し、４℃に一晩放置することにより沈殿を形成させ、上清を回収した。沈殿部にはさらにエタノール１２ｍＬを加えて良く混合後３０分間放置し、遠心分離により上清を集め、先の上清と混合することにより大豆転移レシチン／エタノール溶液を得た。

【００３１】このようにして得た大豆転移レシチン／エタノール溶液２．０ｍＬ（固形分約０．３３ｇ，リン脂質中のＰＳ含量＝３２．５％）に０．１Ｍ酢酸ナトリウム／エタノール溶液を１．０ｍＬ加えて－２０℃で１時間放置し、生じた沈殿（６０ｍｇ）を遠心操作により分離してエタノールで洗浄した（ＰｐｔＮａ－１）。上清を－２０℃で数日間保存した結果、さらに沈澱（７ｍｇ）を生じたので遠心操作により上清（ＳｕｐＮａ，乾固重量＝２２７ｍｇ）と分離し、エタノールで洗浄した（ＰｐｔＮａ－２）。

【００３２】各試料を乾固した後、希釈溶媒（ヘキサン：ジエチルエーテル：イソプロパノール＝２：２：１）に溶解し、薄層クロマトグラフィー（展開溶媒：クロロホルム：メタノール：酢酸＝１３：５：２）で展開してから、Ｄｉｔｔｍｅｒ－Ｌｅｓｔｅｒ試薬によりリン脂質を発色させ、ゲルパターン画像解析システムによりリン脂質含量を定量した。結果を次の表１に示す。

【００３３】表１に示す通り、リン脂質中のＰＳ含量（モル％）は分画前は３２．５％であったのに対して、分画後のＰｐｔＮａでは８１．９％、ＳｕｐＮａでは５．６％であることがわかり、ＰＳが効率よく濃縮できることが確認された。

【００３４】

【表１】

各分画物のリン脂質組成（モル％）

| | ＰＳ | ＰＡ | ＰＣ | ﾘｿﾞＰＣ |
|---|---|---|---|---|
| 大豆転移レシチン | 32.4 | 15.4 | 44.3 | 8.1 |
| PptNa-1 | 81.9 | 14.7 | 3.4 | 0.0 |
| PptNa-2 | 57.2 | 20.0 | 20.5 | 2.3 |
| SupNa | 5.6 | 6.8 | 75.6 | 11.9 |

【００３５】実施例２

実施例１で調製した大豆転移レシチン／エタノール溶液を減圧乾燥し、そのうちの９００ｍｇを５０ｃｃメジウム瓶に取り、クロロホルム２２．５ｍＬとメタノール１５ｍＬの混合液に溶解させた。こうして調製した大豆転移レシチン／クロメタ溶液を４．０ｍＬずつ６．０ｃｃメジウム瓶に分注し、ここに１Ｍ塩類溶液（ａ．塩化リチウム、ｂ．塩化カリウム、ｃ．塩化ナトリウム、ｄ．塩化マグネシウム、ｅ．塩化カルシウム、ｆ．塩化アンモニウム、ｇ．硫酸アンモニウム）を０．８ｍＬ加えた。瓶を振って数回混合した後、静置してクロロホルム層を回収し、その内の１．０ｍＬを秤量した試験管に取り窒素下に乾燥した。

【００３６】こうして得られた乾燥物（約５０ｍｇ，リン脂質含量＝約２５ｍｇ）に対して０．１０ｍｌのジエチルエーテルを加えて溶解し、ここに１．０ｍＬのエタノールを徐々に加えて沈澱を形成させ、懸濁液全体を遠心分離して上清と沈澱とを分けた。

【００３７】エタノール抽出液は２．５倍希釈液を５μＬ、沈澱は全体を２．５ｍＬのクロロホルムに溶解したもの５μＬを薄層板にアプライし、実施例１の条件で展開後、Ｄｉｔｔｍｅｒ－Ｌｅｓｔｅｒ試薬によりリン脂質を発色させゲルパターン画像解析システムによりリン脂質含量を定量した。結果を次の表２に示す。

【００３８】表２に示すように、使用した８種類の塩の中で塩化リチウムが最も成績が良く、１回のエタノール沈澱により純度８０％のＰＳ標品を得ることができた。さらに、塩化リチウムでは全ＰＡのうちの約４分の３がエタノール上清に分画されており、ＰＡの除去効率が際立って良いことがわかった。

【００３９】

【表２】

エタノール沈澱画分リン脂質中のＰＳおよびＰＡ含量（%）

| | ＰＳ | ＰＡ | | ＰＳ | ＰＡ |
|---|---|---|---|---|---|
| 塩化リチウム | 79.8 | 8.1 | 塩化マグネシウム | 52.8 | 18.7 |
| 塩化カリウム | 75.7 | 14.0 | 塩化カルシウム | 53.3 | 19.3 |
| 塩化ナトリウム | 68.2 | 18.8 | 塩化アンモニウム | 殆ど沈澱しない | |
| | | | 硫酸アンモニウム | 殆ど沈澱しない | |

【００４０】　　＊　＊【表３】

エタノール沈澱および上清画分中のリン脂質含量
（画像解析装置により算出されたピーク面積として表示）

| | ＰＳ | | | ＰＡ | | | ＰＣ | | |
|---|---|---|---|---|---|---|---|---|---|
| | 沈澱 | 上清 | 沈／上 | 沈澱 | 上清 | 沈／上 | 沈澱 | 上清 | 沈／上 |
| 塩化リチウム | 9599 | 493 | 19.3 | 1038 | 3046 | 0.34 | 1098 | 11190 | 0.10 |
| 塩化カリウム | 9911 | 1004 | 9.9 | 1959 | 1905 | 1.03 | 1184 | 11652 | 0.10 |
| 塩化ナトリウム | 9487 | 914 | 10.3 | 2242 | 1682 | 1.33 | 1572 | 10409 | 0.15 |

【００４１】なお、全体的な傾向としては陽イオンの原子番号が小さいほどＰＡの除去効率が良く、２価の陽イオンではＰＣも沈澱することがわかった。また、アンモニウム塩の場合にはＰＳを含む大部分のリン脂質が沈殿せず分離には適さなかった。沈殿の成績のよい塩化リチウム、塩化カリウム、塩化ナトリウムに関して、エタノール沈澱および上清画分中のリン脂質含量を表３に示す。

【００４２】実施例３

実施例１で調製した大豆転移レシチン／エタノール溶液を減圧乾燥し、そのうちの９００ｍｇを５０ｃｃメジウム瓶に取り、クロロホルム２２．５ｍＬとメタノール１５ｍＬの混合液に溶解させた。こうして調製した大豆転移レシチン／クロロホルムーメタノール溶液に１M塩化リチウム溶液を８．０ｍＬ加え、瓶を振って数回混合した後、静置してクロロホルム層を回収し減圧乾固した。

【００４３】得られた乾燥物に５．０mLのジエチルエーテルを加えて溶解し、ここに４０ｍＬのエタノールを徐々に加えて溶解し（２０℃）、懸濁液全体を遠心分離して上清（Ｓ１）と沈澱（Ｐ１）とを分離した。Ｐ１に再び５．０mLのジエチルエーテルを加えて溶解し、ここに５０ｍＬのエタノールを徐々に加えて溶解し（２０℃）、懸濁液全体を遠心分離して上清（Ｓ２）と沈澱（Ｐ２）とを分けた。

【００４４】大豆転移レシチンは１０ｍｇ／ｍＬ、Ｐ２（湿潤状態）は２０ｍｇ／ｍＬのクロロホルムに溶解したものを５μＬ，Ｓ１はクロロホルムで２．５倍に希釈したものを５μＬ、そしてＳ２は原液１０μＬを薄層板にアプライし、実施例１の条件で展開後、Ｄｉｔｔｍｅｒ－Ｌｅｓｔｅｒ試薬によりリン脂質を発色させゲルパターン画像解析システムによりリン脂質含量を定量した。

【００４５】エタノール沈澱物をもう一度エタノールで洗浄して得たＰ２画分中のＰＳ含量は９６．９%であった。ＣＭセルロースを用いたイオン交換法における回収率は２０%程度（参考例参照）であったが、本発明による分画法における回収率は約９０%と高く、高純度品を用いた効力評価や作用機構の解明、あるいは医薬品開発に際しての有用な精製手段となり得る。

【００４６】

【表４】

大豆転移ＰＳリチウム塩のエタノール分画

| | ＰＳ | ＰＡ | ＰＣ | LysoＰＣ | その他 |
|---|---|---|---|---|---|
| 大豆転移レシチン | 36.6 | 12.2 | 40.4 | 7.6 | 4.2 |
| Ｐ２ | 96.9 | 1.7 | 0.0 | 0.0 | 1.4 |
| Ｓ１ | 6.8 | 13.7 | 66.7 | 9.3 | 3.4 |
| Ｓ２ | 41.0 | 18.2 | 34.7 | 2.8 | 3.3 |

【００４７】実施例４
実施例１で調製した大豆転移レシチン／エタノール溶液を減圧乾燥し、そのうちの９００ｍｇを５０ｃｃメジウム瓶に取り、クロロホルム２２．５ｍＬとメタノール１５ｍＬの混合液に溶解させた。こうして調製した大豆転移レシチン／クロロホルム－メタノール溶液を４．０ｍＬずつ６．０ｃｃメジウム瓶に分注し、ここに種々のｐＨのクエン酸－クエン酸ナトリウム緩衝液（ｐＨ２．８，３．６，４．１，４．６，５．０）に溶解した２Ｍ食塩水を０．８ｍＬ加えた。瓶を振って数回混合した後、静置してクロロホルム層を回収し、その内の２．５ｍＬを秤量した試験管に取り窒素下に乾燥した。
【００４８】乾燥物（約１２０ｍｇ）に対して０．２ｍＬのジエチルエーテルを加えて溶解し、ここに２．０ｍＬのエタノールを徐々に加えて溶解し、懸濁液全体を遠心分離して上清と沈澱とを分離した。エタノール抽出液は１０倍希釈液を１０μＬ、沈澱は全体を１０．０ｍＬのクロロホルムに溶解したものを５μＬ、薄層板にアプライし、実施例１の条件で展開後Ｄｉｔｔｍｅｒ－Ｌｅｓｔｅｒ試薬によりリン脂質を発色させ、ゲルパターン画像解析システムによりリン脂質含量を定量した。
【００４９】ｐＨ３．６以上の条件ではＰＳもＰＡも共に沈澱画分に回収され（ＰＡ／ＰＳ＝０．２３）、両者を分離することはできなかった。これに対してｐＨ２．８では沈澱に含まれるＰＡ量が相対的に低く（ＰＡ／ＰＳ＝０．１５）、この条件でのエタノール処理を繰り返せばナトリウム塩の状態でＰＳを分画できる可能性が示された。
【００５０】実施例５
ＰＣ含量４０％の大豆レシチン２００ｇにセリン水溶液１９０ｇ（セリン７０ｇ＋水１２０ｇ）とホスホリパーゼＤ（ＰＬＤ－Ｙ１、（株）ヤクルト本社製）の水溶液（２４ｍｇ／ｍＬ）を１０ｍＬ練り込んで５５℃で５時間反応させた結果、リン脂質中のＰＳ含量が４６．７％の反応生成物が得られた。
【００５１】反応生成物５．０ｇにエチルアルコール２０ｍＬを加え４５℃で抽出後、残渣（沈殿）をさらにエチルアルコール５ｍＬで２回抽出した。３回の抽出液を混合し、そのうちの５ｍＬに２５％食塩水０．２０ｍＬを加え、４５℃に加温後、室温に放置して沈殿を形成させた。その結果、上清中のＰＳ含量は乾燥固形分中３．３％であったのに対して、沈殿物では６２．１％であり、ＰＳは沈殿部に効率よく濃縮されることがわかった。
【００５２】実施例６
実施例５で調製した抽出液の混合物５ｍＬに酢酸ナトリウム粉末５０ｍｇを加え４５℃に加温後、室温に放置して沈殿を形成させた。その結果、上清中のＰＳ含量は乾燥固形分中３．５％であったのに対して、沈殿物では６１．８％であり、食塩水を用いた場合と同じく、ＰＳは沈殿部に効率よく濃縮されることがわかった。
【００５３】実施例７
実施例５で調製した抽出液の混合物に対して２５％食塩水を加えてＰＳの不溶物を形成させ、沈殿リン脂質中のＰＳ含量と、沈殿部に回収されるＰＳ量とを測定した。その結果として、食塩添加量と沈殿へのＰＳ回収率及びリン脂質中のＰＳ含量との関係を表５に示す。
【００５４】
【表５】

食塩添加量と沈殿へのＰＳ回収率及びリン脂質中ＰＳ含量との関係

| 食塩添加量 (m moles/g リン脂質) | 沈殿画分へのＰＳ回収率(%) | 沈殿リン脂質中ＰＳ含量(%) | 上清リン脂質中ＰＳ含量(%) |
|---|---|---|---|
| 無添加 | — | — | 43.2 |
| 0.05 | 53.7 | 64.7 | 33.4 |
| 0.15 | 73.8 | 66.7 | 28.2 |
| 0.25 | 80.4 | 69.3 | 18.0 |
| 0.50 | 96.2 | 63.8 | 6.2 |
| 1.25 | 97.4 | 63.5 | 4.3 |
| 2.5 | 96.6 | 62.7 | 6.1 |
| 5 | 97.1 | 59.8 | 6.4 |
| 10 | 95.7 | 55.0 | 10.5 |
| 25 | 97.8 | 47.2 | 12.0 |
| 50 | 97.7 | 46.6 | 13.5 |

【００５５】表５に示す通り、何れの条件でもＰＳは沈殿画分に濃縮されたが、特に抽出液混合物中のリン脂質１ｇあたりに加える食塩の量が１０ミリモル以下の範囲において、沈殿リン脂質中のＰＳ含量は５５％以上であり、沈殿部にＰＳが効率よく濃縮されていた。一方、食塩添加量が０．０５ミリモル以下の場合には、沈殿へのＰＳの回収率は６０％以下であり、４０％以上が上清部に存在していた。以上の結果から、何れの添加量でもＰＳを沈殿に濃縮することが可能であるが、アルコールに溶解したリン脂質１ｇに対する食塩の添加量が０．１５～１０ミリモルの範囲が特に実用に適した添加量と考えられた。

【００５６】参考例　ＣＭ－セルロース・カラムクロマトグラフィーによる精製

牛脳からのＰＳの精製例（新生化学実験講座４、脂質II リン脂質、ｐ．１２７）に準じて実施例１で得た大豆転移レシチンからＰＳを精製することを試みた。

【００５７】（１）　Ｎａ＋型ＣＭ－セルロースの調製：ＣＭ－５２（Ｗｈａｔｍａｎｎ社、膨潤型）５０ｇを０．５Ｎ水酸化ナトリウム５００ｍＬ中に徐々に加えて約３０分間静置した後に吸引濾過した。濾液が中性になるまで蒸留水で洗浄（５００ｍＬ×２回）した後、０．５Ｎ塩酸５００ｍＬを流し蒸留水で濾液が中性になるまで再度洗浄（５００ｍＬ×２回）した。このゲルを０．５Ｎ水酸化ナトリウム５００ｍＬ中にかきまぜながら加え３０分間静置したのち、蒸留水で中性になるまで洗浄（５００ｍＬ×１回）し、さらにメタノール５００ｍＬで洗浄後、最終的にメタノール懸濁液として室温に保存した。

【００５８】（２）　クロマトグラフィー：（１）で調整したＣＭ－５２を内径３０ｍｍのカラムに充填（ベッド体積７０ｍＬ）し、クロロホルムを５００ｍＬ流してコンディショニングした後、実施例１の大豆転移レシチン１．０ｇを１０ｍＬのクロロホルムに溶解して沈澱を除いた溶液をカラムにアプライした後、クロロホルムで溶出させ、２００ｍＬのクロロホルム溶出画分（Ｆｒ．１）を得た。

【００５９】更に、クロロホルム－メタノール（８５：１５，ｖ／ｖ）４００ｍＬ（Ｆｒ．２＋３）、クロロホルム－メタノール（７５：２５，ｖ／ｖ）４００ｍＬ（Ｆｒ．４＋５）、クロロホルム－メタノール（６５：３５，ｖ／ｖ）４００ｍＬ（Ｆｒ．６＋７）、クロロホルム－メタノール（５０：５０，ｖ／ｖ）４００ｍＬ（Ｆｒ．８＋９）を流して２００ｍＬずつを分画し、シリカゲル薄層クロマトグラフィーによりリン脂質を分析した。

【００６０】その結果、クロロホルム溶出画分（Ｆｒ．１）にはほとんどリン脂質が検出されなかったが、クロロホルム－メタノール（８５：１５，ｖ／ｖ）画分の前半（Ｆｒ．２）には白濁状態でアプライしたエタノール沈澱物とほぼ同じ組成の物が０．２９ｇ（乾燥重量）溶出された。クロロホルム－メタノール（７５：２５，ｖ／ｖ）画分（Ｆｒ．４＋５）にはＰＣは含まれなかったがＰＳとＰＡの両方が含まれており、特にＰＡが多く溶出されていた。そして、クロロホルム－メタノール（６５：３５，ｖ／ｖ）画分の前半（Ｆｒ．６）にはまだ８．０％のＰＡが含まれていたが、後半（Ｆｒ．７）以降にはＰＡが含まれておらず、ＰＳ以外には原点にわずかの発色が見られるのみで純度は９７．７％であった。なお、クロロホルム－メタノール（５０：５０，ｖ／ｖ）画分の前半（Ｆｒ．８）には相当量のＰＳが含まれていたが、後半（Ｆｒ．９）にはほとんど溶出物がなかった。

【００６１】以上の結果から、大豆転移レシチンからも牛脳分画物とほぼ同様の条件の陽イオン交換クロマトによりＰＳを精製できることが明らかになったが、回収率は極めて低く（Ｆｒ．７以降のみを回収した場合，２０％程度）、大量の精製ＰＳを得るには適さない方法であることがわかった。

【００６２】

13　　14

【発明の効果】本発明は以上説明した通り、天然あるいは人工的に調製されたリン脂質混合物からＰＳを簡便に＊＊濃縮する分画法を得ることができるという効果がある。

---

フロントページの続き

(72)発明者　工藤　聰
東京都港区東新橋１丁目１番19号　株式会社ヤクルト本社内

Ｆターム(参考)　4H050 AA02 AD15 AD17 AD30 BB14 BE61

[JP,2002-241385,A]

---

CLAIMS DETAILED DESCRIPTION TECHNICAL FIELD PRIOR ART EFFECT OF THE INVENTION TECHNICAL PROBLEM MEANS EXAMPLE

---

[Translation done.]

* NOTICES *



1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

---

## CLAIMS

---

[Claim(s)]
[Claim 1]A fractionation method of phosphatidylserine making phosphatidylserine insolubilize and separating this insoluble part by adding metal salt in this solution after dissolving a phospholipid mixture containing phosphatidylserine in alcohols.
[Claim 2]A fractionation method of the phosphatidylserine according to claim 1 using one sort chosen from lithium salt, potassium salt, and sodium salt, or two sorts or more as said metal salt.
[Claim 3]A fractionation method of the phosphatidylserine according to claim 1 or 2 characterized by using a lithium chloride, potassium chloride, or sodium chloride as said metal salt.
[Claim 4]A fractionation method of the phosphatidylserine according to any one of

claims 1 to 3 characterized by using ethyl alcohol as said alcohols.

---

[Translation done.]

## DETAILED DESCRIPTION

---

[Detailed Description of the Invention]
[0001]
[Field of the Invention]This invention relates to the fractionation method for condensing phosphatidylserine from a phospholipid mixture.
[0002]
[Description of the Prior Art]The use as the treating agent and surface-active agent of an immune disease besides the cerebral function reforming agent [ phosphatidylserine / (it is hereafter indicated as "PS".) ] aiming at prevention, a therapy, etc. of dementia is expected. This PS is contained in the brain and muscles of an animal, and also it can be artificially manufactured by the transphosphatidylation which uses a chemosynthesis method and phospholipase D.
[0003]Since PS is mainly used as medicine, foodstuffs, and cosmetics, it is important for it to carry out fractionation of the PS from said natural product, a reactant, etc., and to raise PS content. However, since it is difficult to manufacture a product with many PS contents cheaply, when using it, concentration (refining) of PS is needed [ there are few amounts of PS contained in the brain and muscles of an animal, and ], also when it manufactures by a chemosynthesis method or transphosphatidylation in many cases.
[0004]As a method of condensing PS (refining), the fractionation and column chromatography by a solvent have been used conventionally. However, it is difficult to obtain PS purity sufficient by just solvent fractionation, and, on the other hand, complicated operation like chromatography had a problem in respect of a cost aspect and workability. For this reason, development of the method of condensing PS cheaply and simple is desired.
[0005]Since especially PS is difficult to separate from other phospholipid (PC), i.e., phosphatidylcholine, phosphatidylethanolamine (PE), phosphatidylinositol (PI),

phosphatidic acid (PA), etc., To establish the method of condensing PS cheaply from the phospholipid mixture containing these is desired.
[0006]
[Problem(s) to be Solved by the Invention]This invention persons dissolved the phospholipid mixture containing PS in alcohols, as a result of repeating research wholeheartedly, in order to solve an aforementioned problem, and they made PS precipitate by adding metal salt or its solution further, and it found out that it could condense to a precipitating part.
[0007]An object of this invention is to acquire the fractionation method which condenses PS simple from the phospholipid mixture prepared nature or artificially.
[0008]
[Means for Solving the Problem]After a fractionation method of PS concerning an invention indicated to claim 1 dissolves a phospholipid mixture containing PS in alcohols, by adding metal salt in this solution, it makes PS insolubilize and separates this insoluble part.
[0009]One sort chosen from lithium salt, potassium salt, and sodium salt or two sorts or more are used for a fractionation method of PS concerning an invention indicated to claim 2 as the metal salt according to claim 1.
[0010]A lithium chloride, potassium chloride, or sodium chloride is used for a fractionation method of PS concerning an invention indicated to claim 3 as the metal salt according to claim 1 or 2.
[0011]Ethyl alcohol is used for a fractionation method of PS concerning an invention indicated to claim 4 as the alcohols according to any one of claims 1 to 3.
[0012]
[Embodiment of the Invention]In this invention, after dissolving the phospholipid mixture containing PS in alcohols, insoluble matters (a precipitating part, a flocculation part, etc.) are separated and condensed by making PS insolubilize (precipitate, condensation, etc.) by simple operation of adding metal salt in this solution. PS can be condensed simple from the phospholipid mixture prepared nature or artificially by this.
[0013]As a phospholipid mixture containing PS used for this invention, as long as it is a mixture containing PS, such as a thing which refined the extract or this extract from a natural product and a natural product, or synthetic phospholipid, any may be used. Specifically, a phospholipid mixture, a solvent extraction thing of a bovine brain, etc.

which were prepared by a soybean lecithin, rapeseed lecithin, yolk lecithin, corn lecithin or cottonseed lecithin, a chemosynthesis method, or transphosphatidylation are mentioned. Especially, if the phospholipid mixture containing PS prepared by transphosphatidylation is used, the degree of enrichment at the time of adding metal salt is high, and preferred also from the ease of carrying out and cost aspect of reservation of a raw material.

[0014]As metal salt used for this invention, lithium salt, sodium salt, Metal salt, such as potassium salt, calcium salt, and magnesium salt, or the natural product which contains these abundantly, For example, although salt, bittern, saline water, dolomite, edible mother-of-pearl powder, etc. may use any, it is preferred from a point of condensation efficiency to use lithium salt, sodium salt, or potassium salt, and especially a lithium chloride, sodium chloride, or potassium chloride is preferred. These metal salt can be used combining one sort or two sorts or more.

[0015]Especially if the addition of these metal salt is the quantity which may settle PS, it will not be limited, but it is [ the recovery rate of PS and its PS content under precipitate ] preferred from a high point per [ phospholipid 1g ] and that it is 0.5 to 5 millimol especially 0.15 to 10 millimol.

[0016]If it is alcohols which can dissolve a phospholipid mixture as alcohols used for this invention, all will be used suitably, but. Lower alcohol, such as methyl alcohol, ethyl alcohol, butyl alcohol, propyl alcohol, and isopropyl alcohol, is especially preferred. Although these mixtures can also be used, it is easy to use it for foodstuffs, and since ethyl alcohol also has few problems in a safety aspect, especially the thing for which this is used is preferred [ ethyl alcohol ].

[0017]Although the concentration in particular at the time of dissolving a phospholipid mixture in alcohols is not limited, it is preferred to consider it as the above quantity that this mixture can dissolve thoroughly, and it is preferred from PS condensation efficiency or a point of operativity to consider it as 2 to 20% especially 1 to 50% to the weight of alcohols.

[0018]In this invention, fractionation of PS from a phospholipid mixture can be performed as follows, for example. First, it is prepared by a transphosphatidylation method etc. and the phospholipid mixture which contains phospholipid other than PS, such as PC, PE, or PA, in an ingredient is dissolved in alcohols, such as ethyl alcohol. At this time, the conditions in particular of the dissolution of a melting temperature etc.

are not limited, but are doubled with the kinds of ingredient of a mixture, those quantity, etc., and should just choose and use suitable conditions.

[0019]In this way, in the solution obtained, although phospholipid, such as PS, PC, PA, is extracted by the solvent layer, depending on the case, some insoluble components generate it. For this reason, metal salt is added after removing insoluble components (a sediment, an aggregate, etc.) from a solvent by centrifugal separation, filtration, or other means. When a little PS remains also in an insoluble component, the extracting processing by said alcohols is good in an abundance repetition line.

[0020]Subsequently, to an alcohol solution, metal salt is added and fractionation of the PS extracted by the solvent layer is carried out. That is, although the great portion of phospholipid other than PS in a solvent layer does not precipitate by addition of metal salt, either, since the most precipitates, PS can condense PS by collecting these. Even if it adds metal salt with powder at this time, it may add, after melting in solvents, such as water and alcohol. What is necessary is not to limit the various conditions in particular in that case, either, but to double with the kinds of ingredient of a mixture, those quantity, etc., and just to choose suitable conditions. What is necessary is to specifically hold 30 minutes or more at 10 ** - 30 **, and just to make PS insolubilize.

[0021]PS insolubilized by addition of metal salt is recoverable by centrifugal separation, filtration, settlement separation, or other means. It is also possible to refine further by the publicly known refining means, for example, column chromatography etc., or other means. Since the content of other phospholipid is falling notably, PS concentrate of this invention can be performed comparatively simple [ such a refining means ].

[0022]PS concentrate of this invention can be prescribed for the patient with the gestalt of drugs, foodstuffs, cosmetics, etc. For example, if it is a case where it uses with the gestalt of the drugs which solicit the physiology effect of phospholipid, a supplement, etc., it can administer orally as liquid preparations, such as solid preparations, such as a capsule, a granule, a tablet, and powder medicine, or syrups. Even if it is not an orally administered drug, it is also possible to prescribe a medicine for the patient with parenteral gestalten, such as injections, skin external preparations, and a rectum administration agent.

[0023]At the time of manufacture of each pharmaceutical preparation, milk sugar, starch, crystalline cellulose, calcium lactate, Excipients, such as magnesium aluminometasilicate and a silicic acid anhydride, white soft sugar, Binding materials,

such as hydroxypropylcellulose and a polyvinyl pyrrolidone, What is necessary is just to use suitably lubricant, such as disintegrator, such as carboxymethyl cellulose and carboxymethyl-cellulose calcium, magnesium stearate, talc, monoglyceride, and a sucrose fatty acid ester, and the other ingredients which may be permitted as medicine and foodstuffs.

[0024]What is necessary is to add what carried out purification treatment of the PS concentrate obtained by the method of this invention as it is or suitably in eating-and-drinking articles, such as fats and oils, a hard candy, fermented milk, a candy, a seasoning, and fish flour, and just to manufacture using a conventional method, in expecting the same physiology effect and using with a common foodstuffs gestalt (gestalt of "clear foodstuffs").

[0025]On the occasion of use with the gestalt of these drugs, foodstuffs, etc., the phospholipid composition in which PS obtained by the method of this invention was condensed can be blended suitably. what is necessary is just to blend suitably the quantity which is a grade which can acquire the effect and problems, such as superfluous ingestion, do not produce, and the quantity the ingestion which is 50 mg - about 1000mg/day is expected to be, if it is a case where the physiology effect of PS is solicited

[0026]As for phospholipid of this invention, it is preferred to use as an emulsifier and to add 0.01 to 10% to drugs, foodstuffs, cosmetics, etc. in that case.

[0027]

[Example]Although an example is given to below and this invention is explained to it, this invention is not limited to these.

[0028]while taking 10.0 g of example 1 soybean lecithins (PC80: made by clo KURAN), and the soybean oil 2.0g into a 100-cc medium bottle, adding 90.0 g (100mL) ethyl acetate here and agitating with a stirrer -- warming -- it dissolved. **** picking and 0.1M sodium phosphate buffer solution (pH 7.0) 5.8mL was added, and L-serine 1.2g and PLD-Y1 (Made by Yakult Honsha) 1,500 unit were dissolved.

[0029]The medium bottle containing PC80 solution whole quantity is kept warm at 50 **, the whole quantity of the solution (L-serine+PLD-Y1) which kept it warm at 50 ** is added here, and a reaction is started, and it was made to react at 50 ** for 5 hours, stirring gently with a stirrer. After ice-cooling for 30 minutes, settling phospholipid and collecting, in boiling water, it was neglected for 20 minutes and the enzyme was

deactivated.

[0030]Ethanol 40mL could be added to the collected phospholipid layer, and it mixed, and by neglecting it at 4 ** overnight, precipitate was made to form and supernatant liquid was collected. Ethanol 12mL could be further added to the precipitating part, it was neglected for 30 minutes after mixing, supernatant liquid was collected by centrifugal separation, and soybean transition lecithin / ethanol solution was obtained by mixing with previous supernatant liquid.

[0031]Thus, add 0.1M sodium acetate / ethanol solution to the obtained soybean transition lecithin / ethanol solution 2.0mL (about 0.33 g of solid content, PS content in phospholipid = 32.5%) 1.0 mL, and it is neglected at −20 ** for 1 hour, Centrifugal operation separated the formed precipitate (60 mg), and it washed by ethanol (PptNa−1). As a result of saving supernatant liquid for several days at −20 **, since the precipitate (7 mg) was produced further, centrifugal operation separated with supernatant liquid (SupNa, hardening−by−drying weight = 227 mg), and it washed by ethanol (PptNa−2).

[0032]After hardening each sample by drying, it dissolves in a diluent solvent (hexane: diethylether : isopropanol =2:2:1), After developing with thin layer chromatography (chloroform: developing solvent : methanol : acetic acid =13:5:2), under [ a fixed quantity / analysis system / gel pattern image / content / phospholipid / it makes phospholipid color with a Dittmer−Lester reagent and ]. A result is shown in the next table 1.

[0033]It was checked that, as for before fractionation, it turns out to having been 32.5% that it is [ which it is 81.9% in PptNa after fractionation ] 5.6% in SupNa, and PS can condense PS content (mol %) in phospholipid efficiently as shown in Table 1.

[0034]

[Table 1]

[0035]Reduced pressure drying of the soybean transition lecithin / the ethanol solution prepared in example 2 Example 1 was carried out, 900 mg of them was taken into the 50-cc medium bottle, and it was made to dissolve in the mixed liquor of chloroform 22.5mL and methanol 15mL. In this way, the prepared soybean transition lecithin / clo meta-solution are poured distributively into every 4.0 mL 6.0-cc medium bottle, 1M saline (a. a lithium chloride, b. potassium chloride, c. sodium chloride, d. magnesium chloride, e. calcium chloride, f. ammonium chloride, g. ammonium sulfate) was added here 0.8 mL. After shaking the bottle and mixing several times, it settled, chloroform layers were collected, and it took in the test tube which carried out weighing of the 1.0mL of them, and dried under nitrogen.

[0036]In this way, to the obtained dry matter (about 50 mg, a phospholipid content = about 25 mg), added 0.10-ml diethylether and dissolved, and added the ethanol of 1.0mL here gradually, precipitation was made to form, the whole suspension was centrifuged, and supernatant liquid and precipitation were divided.

[0037]An ethanol extract applies 5micro of things L by which 5microL and precipitation dissolved the whole for the diluent in chloroform of 2.5mL 2.5 times to a laminated plate, Under [ a fixed quantity / analysis system / gel pattern image / content / phospholipid / it makes phospholipid color after deployment and with a Dittmer-Lester reagent on condition of Example 1 and ]. A result is shown in the next table 2.

[0038]As shown in Table 2, the lithium chloride was the highest-achieving of eight kinds of the used salts, and PS preparation of 80% of purity was able to be obtained by the ethanol precipitation which is 1 time. It turned out that fractionation of about 3/4 of all the PA is carried out to ethanol supernatant liquid, and the removal efficiency of PA may be conspicuous for a lithium chloride.

[0039]

[Table 2]

[0040]
[Table 3]

[0041]The removal efficiency of PA was so good that the atomic number of the positive ion was small as an overall trend, and it turned out in the divalent positive ion that PC also precipitates. Phospholipid of most which contains PS in the case of ammonium salt did not precipitate, and it was not suitable for separation. The phospholipid content in ethanol precipitation and a supernatant liquid fraction is shown in Table 3 about the high-achieving lithium chloride of precipitate, potassium chloride, and sodium chloride.

[0042]Reduced pressure drying of the soybean transition lecithin / the ethanol solution prepared in example 3 Example 1 was carried out, 900 mg of them was taken into the 50-cc medium bottle, and it was made to dissolve in the mixed liquor of chloroform 22.5mL and methanol 15mL. In this way, 1M lithium-chloride solution was added to the prepared soybean transition lecithin / chloroform methanol solution 8.0 mL, after

shaking the bottle and mixing several times, it settled, and chloroform layers were collected and decompression hardening by drying was carried out.

[0043]Diethylether of 5.0mL was added to the obtained dry matter, and it dissolved in it, and the ethanol of 40mL was added here gradually, it dissolved (20 **), the whole suspension was centrifuged, and supernatant liquid (S1) and precipitation (P1) were separated. Diethylether of 5.0mL was again added to P1, and it dissolved in it, and the ethanol of 50mL was added here gradually, it dissolved (20 **), the whole suspension was centrifuged, and supernatant liquid (S2) and precipitation (P2) were divided.

[0044]That to which 5microL and S1 diluted with chloroform what soybean transition lecithin dissolved in 10 mg/mL, and dissolved P2 (damp or wet condition) in chloroform of 20 mg/mL 2.5 times 5microL, And apply undiluted solution 10muL to a laminated plate, phospholipid is made to color after deployment and with a Dittmer–Lester reagent on condition of Example 1, and S2 is the bottom about a fixed quantity of phospholipid contents by a gel pattern image analysis system.

[0045]PS content in P2 fraction which washed and obtained the ethanol precipitation thing by ethanol once again was 96.9%. Although the recovery rate in the ionic exchange method using CM cellulose was about (refer to reference example) 20%, the recovery rate in the fractionation method by this invention can serve as a useful refining means for about 90%, the effect evaluation it is high and using the high grade article, a break through of the mechanism of action, or drugs development.

[0046]

[Table 4]

[0047]Reduced pressure drying of the soybean transition lecithin / the ethanol solution prepared in example 4 Example 1 was carried out, 900 mg of them was taken into the

50-cc medium bottle, and it was made to dissolve in the mixed liquor of chloroform 22.5mL and methanol 15mL. In this way, the prepared soybean transition lecithin / chloroform methanol solution were poured distributively into every 4.0 mL 6.0-cc medium bottle, and 2M salt solution which dissolved in the citrate-sodium-acid-citrate buffer solution (pH 2.8, 3.6, 4.1, 4.6, 5.0) of various pH was added here 0.8 mL. After shaking the bottle and mixing several times, it settled, chloroform layers were collected, and it took in the test tube which carried out weighing of the 2.5mL of them, and dried under nitrogen.

[0048]To the dry matter (about 120 mg), diethylether of 0.2mL was added and it dissolved, and the ethanol of 2.0mL was added here gradually, it dissolved, the whole suspension was centrifuged, and supernatant liquid and precipitation were separated. An ethanol extract that by which 10microL and precipitation dissolved the whole for the diluent in chloroform of 10.0mL 10 times 5microL, Under [ a fixed quantity / analysis system / gel pattern image / content / phospholipid / apply to a laminated plate, and it makes phospholipid color with an after-deployment Dittmer-Lester reagent on condition of Example 1 and ].

[0049]On pH 3.6 or more conditions, both PS and PA were collected by the precipitation fraction (PA/PS=0.23), and were not able to separate both. On the other hand, a possibility that it could carry out fractionation of the PS in the state of sodium salt low (PA/PS=0.15) relatively if the amount of PA contained in precipitation repeats the ethanol treatment in this condition was shown by pH 2.8.

[0050]The result of having scoured the serine solution 190g (serine 70g+ water 120g) and the solution (24 mg/mL) of phospholipase D (PLD-Y1, Yakult Honsha Make) to the soybean lecithin 200g of 40% of the example 5PC content 10 mL, and having made them reacting to it at 55 ** for 5 hours, The resultant whose PS content in phospholipid is 46.7% was acquired.

[0051]Ethyl alcohol 20mL was added to the resultant 5.0g, and ethyl alcohol 5mL extracted residue (precipitate) twice further after extraction at 45 **. 3 times of extracts are mixed, salt solution 0.20mL is added to 5mL of them 25%, it was neglected to the room temperature and precipitate was made to form after warming at 45 **. As a result, it turned out that it is 62.1% in a sediment and PS is efficiently condensed by the precipitating part to PS content in supernatant liquid having been 3.3% among the dry solid.

[0052]50 mg of sodium acetate powder is added to mixture 5mL of the extract prepared in example 6 Example 5, it was neglected to the room temperature and precipitate was made to form after warming at 45 **. As a result, it turned out that PS is efficiently condensed by the precipitating part as well as the case where are 61.8% in a sediment and a salt solution is used to PS content in supernatant liquid having been 3.5% among the dry solid.

[0053]Added the salt solution 25% to the mixture of an extract prepared in example 7 Example 5, the insoluble matter of PS was made to form, and PS content in precipitate phospholipid and the amount of PS collected by the precipitating part were measured. As the result, the relation between a salt addition, PS recovery rate to precipitate, and PS content in phospholipid is shown in Table 5.

[0054]

[Table 5]

[0055]PS was condensed by the precipitate fraction also on condition of any as shown in Table 5, but the quantity of the salt especially added to per [ phospholipid 1g in an extract mixture ] of PS content in precipitate phospholipid is not less than 55% in the range of 10 or less millimols, and PS was efficiently condensed by the precipitating part. On the other hand, when a salt addition was 0.05 or less millimols, the recovery rate of PS to precipitate is 60% or less, and not less than 40% existed in the supernatant liquid part. Although it was possible to have condensed PS from the above result to

precipitate with any addition, the addition of the salt to the phospholipid 1g which dissolved in alcohol was considered to be the addition to which especially the range of 0.15 to 10 millimol was suitable for practical use.

[0056]Reference example It tried to refine PS from the soybean transition lecithin obtained in Example 1 according to the example of refining of PS from the refining bovine brains by CM cellulose column chromatography (the new chemical experiment lecture 4, lipid II phospholipid, p.127).

[0057](1) Preparation of Na+ type CM cellulose : suction **** was carried out, after adding CM-52 (Whatmann, swollen type) 50g gradually and settling it for about 30 minutes into 0.5N sodium hydroxide 500mL. It washed again after distilled water washed until **** became neutrality (500mLx2 time) until it passed 0.5N chloride 500mL and **** became neutrality with distilled water (500mLx2 time). After settling for 30 minutes in addition, stirring this gel in 0.5N sodium hydroxide 500mL, it washed until it became neutrality with distilled water (500mLx1 time), and saved as methanol suspension eventually after washing by methanol 500mL further at the room temperature.

[0058](2) A column 30 mm in inside diameter is filled up with CM-52 adjusted by chromatography: (1) (bed volume 70mL), After having dissolved the soybean transition lecithin 1.0g of Example 1 in chloroform of 10mL after 500-mL-pouring chloroform and carrying out a conditioning, and applying the solution except precipitation to a column, you made it eluted under chloroform and the chloroform eluate fraction (Fr.1) of 200mL was obtained.

[0059]Chloroform methanol (85:15, v/v) 400mL (Fr.2+3), Chloroform methanol (75:25, v/v) 400mL (Fr.4+5), Chloroform methanol (65:35, v/v) 400mL (Fr.6+7) and chloroform methanol (50:50, v/v) 400mL (Fr.8+9) were passed, fractionation of every 200 mL was carried out, and silica gel thin layer chromatography analyzed phospholipid.

[0060]As a result, although phospholipid was hardly detected by the chloroform eluate fraction (Fr.1), 0.29g (dry weight) elution of the thing of the almost same presentation as the ethanol precipitation thing applied by the cloudy state was carried out in the first half (Fr.2) of a chloroform methanol (85:15, v/v) fraction. Although PC was not contained in the chloroform methanol (75:25, v/v) fraction (Fr.4+5), both PS and PA are contained, and it was mostly eluted especially in PA. And purity was [ that PA is not contained on and after the second half (Fr.7), but coloring slight at the starting point in

addition to PS is only seen, and ] 97.7% although 8.0% of PA was still contained in the first half (Fr.6) of a chloroform methanol (65:35, v/v) fraction. Although a considerable amount of PS was contained in the first half (Fr.8) of a chloroform methanol (50:50, v/v) fraction, there was almost no effluent in the second half (Fr.9).

[0061]Although it became clear from the above result that PS can be refined also from soybean transition lecithin by the cation-exchange chromatography of the almost same conditions as a bovine brain fraction, It turned out that it is the method of not being suitable for a recovery rate obtaining a lot of refining PS very low (it is about 20% when Fr.7 or subsequent ones are collected).

[0062]

[Effect of the Invention]This invention is effective in the ability to acquire the fractionation method which condenses PS simple from the phospholipid mixture prepared nature or artificially as it was explained above.

---

[Translation done.]

## TECHNICAL FIELD

---

[Field of the Invention]This invention relates to the fractionation method for condensing phosphatidylserine from a phospholipid mixture.

---

[Translation done.]

## PRIOR ART

---

[Description of the Prior Art]The use as the treating agent and surface-active agent of an immune disease besides the cerebral function reforming agent [ phosphatidylserine / (it is hereafter indicated as "PS".) ] aiming at prevention, a therapy, etc. of dementia is expected. This PS is contained in the brain and muscles of an animal, and also it can

be artificially manufactured by the transphosphatidylation which uses a chemosynthesis method and phospholipase D.

[0003]Since PS is mainly used as medicine, foodstuffs, and cosmetics, it is important for it to carry out fractionation of the PS from said natural product, a reactant, etc., and to raise PS content. However, since it is difficult to manufacture a product with many PS contents cheaply, when using it, concentration (refining) of PS is needed [ there are few amounts of PS contained in the brain and muscles of an animal, and ], also when it manufactures by a chemosynthesis method or transphosphatidylation in many cases.

[0004]As a method of condensing PS (refining), the fractionation and column chromatography by a solvent have been used conventionally. However, it is difficult to obtain PS purity sufficient by just solvent fractionation, and, on the other hand, complicated operation like chromatography had a problem in respect of a cost aspect and workability. For this reason, development of the method of condensing PS cheaply and simple is desired.

[0005]Since especially PS is difficult to separate from other phospholipid (PC), i.e., phosphatidylcholine, phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), etc., To establish the method of condensing PS cheaply from the phospholipid mixture containing these is desired.

---

[Translation done.]

## EFFECT OF THE INVENTION

---

[Effect of the Invention]This invention is effective in the ability to acquire the fractionation method which condenses PS simple from the phospholipid mixture prepared nature or artificially as it was explained above.

---

[Translation done.]

## TECHNICAL PROBLEM

[Problem(s) to be Solved by the Invention]This invention persons dissolved the phospholipid mixture containing PS in alcohols, as a result of repeating research wholeheartedly, in order to solve an aforementioned problem, and they made PS precipitate by adding metal salt or its solution further, and it found out that it could condense to a precipitating part.

[0007]An object of this invention is to acquire the fractionation method which condenses PS simple from the phospholipid mixture prepared nature or artificially.

[Translation done.]

## MEANS

[Means for Solving the Problem]After a fractionation method of PS concerning an invention indicated to claim 1 dissolves a phospholipid mixture containing PS in alcohols, by adding metal salt in this solution, it makes PS insolubilize and separates this insoluble part.

[0009]One sort chosen from lithium salt, potassium salt, and sodium salt or two sorts or more are used for a fractionation method of PS concerning an invention indicated to claim 2 as the metal salt according to claim 1.

[0010]A lithium chloride, potassium chloride, or sodium chloride is used for a fractionation method of PS concerning an invention indicated to claim 3 as the metal salt according to claim 1 or 2.

[0011]Ethyl alcohol is used for a fractionation method of PS concerning an invention indicated to claim 4 as the alcohols according to any one of claims 1 to 3.

[0012]

[Embodiment of the Invention]In this invention, after dissolving the phospholipid mixture containing PS in alcohols, insoluble matters (a precipitating part, a flocculation

part, etc.) are separated and condensed by making PS insolubilize (precipitate, condensation, etc.) by simple operation of adding metal salt in this solution. PS can be condensed simple from the phospholipid mixture prepared nature or artificially by this.

[0013]As a phospholipid mixture containing PS used for this invention, as long as it is a mixture containing PS, such as a thing which refined the extract or this extract from a natural product and a natural product, or synthetic phospholipid, any may be used. Specifically, a phospholipid mixture, a solvent extraction thing of a bovine brain, etc. which were prepared by a soybean lecithin, rapeseed lecithin, yolk lecithin, corn lecithin or cottonseed lecithin, a chemosynthesis method, or transphosphatidylation are mentioned. Especially, if the phospholipid mixture containing PS prepared by transphosphatidylation is used, the degree of enrichment at the time of adding metal salt is high, and preferred also from the ease of carrying out and cost aspect of reservation of a raw material.

[0014]As metal salt used for this invention, lithium salt, sodium salt, Metal salt, such as potassium salt, calcium salt, and magnesium salt, or the natural product which contains these abundantly, For example, although salt, bittern, saline water, dolomite, edible mother-of-pearl powder, etc. may use any, it is preferred from a point of condensation efficiency to use lithium salt, sodium salt, or potassium salt, and especially a lithium chloride, sodium chloride, or potassium chloride is preferred. These metal salt can be used combining one sort or two sorts or more.

[0015]Especially if the addition of these metal salt is the quantity which may settle PS, it will not be limited, but it is [ the recovery rate of PS and its PS content under precipitate ] preferred from a high point per [ phospholipid 1g ] and that it is 0.5 to 5 millimol especially 0.15 to 10 millimol.

[0016]If it is alcohols which can dissolve a phospholipid mixture as alcohols used for this invention, all will be used suitably, but. Lower alcohol, such as methyl alcohol, ethyl alcohol, butyl alcohol, propyl alcohol, and isopropyl alcohol, is especially preferred. Although these mixtures can also be used, it is easy to use it for foodstuffs, and since ethyl alcohol also has few problems in a safety aspect, especially the thing for which this is used is preferred [ ethyl alcohol ].

[0017]Although the concentration in particular at the time of dissolving a phospholipid mixture in alcohols is not limited, it is preferred to consider it as the above quantity that this mixture can dissolve thoroughly, and it is preferred from PS condensation

efficiency or a point of operativity to consider it as 2 to 20% especially 1 to 50% to the weight of alcohols.

[0018]In this invention, fractionation of PS from a phospholipid mixture can be performed as follows, for example. First, it is prepared by a transphosphatidylation method etc. and the phospholipid mixture which contains phospholipid other than PS, such as PC, PE, or PA, in an ingredient is dissolved in alcohols, such as ethyl alcohol. At this time, the conditions in particular of the dissolution of a melting temperature etc. are not limited, but are doubled with the kinds of ingredient of a mixture, those quantity, etc., and should just choose and use suitable conditions.

[0019]In this way, in the solution obtained, although phospholipid, such as PS, PC, PA, is extracted by the solvent layer, depending on the case, some insoluble components generate it. For this reason, metal salt is added after removing insoluble components (a sediment, an aggregate, etc.) from a solvent by centrifugal separation, filtration, or other means. When a little PS remains also in an insoluble component, the extracting processing by said alcohols is good in an abundance repetition line.

[0020]Subsequently, to an alcohol solution, metal salt is added and fractionation of the PS extracted by the solvent layer is carried out. That is, although the great portion of phospholipid other than PS in a solvent layer does not precipitate by addition of metal salt, either, since the most precipitates, PS can condense PS by collecting these. Even if it adds metal salt with powder at this time, it may add, after melting in solvents, such as water and alcohol. What is necessary is not to limit the various conditions in particular in that case, either, but to double with the kinds of ingredient of a mixture, those quantity, etc., and just to choose suitable conditions. What is necessary is to specifically hold 30 minutes or more at 10 ** - 30 **, and just to make PS insolubilize.

[0021]PS insolubilized by addition of metal salt is recoverable by centrifugal separation, filtration, settlement separation, or other means. It is also possible to refine further by the publicly known refining means, for example, column chromatography etc., or other means. Since the content of other phospholipid is falling notably, PS concentrate of this invention can be performed comparatively simple [ such a refining means ].

[0022]PS concentrate of this invention can be prescribed for the patient with the gestalt of drugs, foodstuffs, cosmetics, etc. For example, if it is a case where it uses with the gestalt of the drugs which solicit the physiology effect of phospholipid, a supplement, etc., it can administer orally as liquid preparations, such as solid

preparations, such as a capsule, a granule, a tablet, and powder medicine, or syrups. Even if it is not an orally administered drug, it is also possible to prescribe a medicine for the patient with parenteral gestalten, such as injections, skin external preparations, and a rectum administration agent.

[0023]At the time of manufacture of each pharmaceutical preparation, milk sugar, starch, crystalline cellulose, calcium lactate, Excipients, such as magnesium aluminometasilicate and a silicic acid anhydride, white soft sugar, Binding materials, such as hydroxypropylcellulose and a polyvinyl pyrrolidone, What is necessary is just to use suitably lubricant, such as disintegrator, such as carboxymethyl cellulose and carboxymethyl-cellulose calcium, magnesium stearate, talc, monoglyceride, and a sucrose fatty acid ester, and the other ingredients which may be permitted as medicine and foodstuffs.

[0024]What is necessary is to add what carried out purification treatment of the PS concentrate obtained by the method of this invention as it is or suitably in eating-and-drinking articles, such as fats and oils, a hard candy, fermented milk, a candy, a seasoning, and fish flour, and just to manufacture using a conventional method, in expecting the same physiology effect and using with a common foodstuffs gestalt (gestalt of "clear foodstuffs").

[0025]On the occasion of use with the gestalt of these drugs, foodstuffs, etc., the phospholipid composition in which PS obtained by the method of this invention was condensed can be blended suitably. what is necessary is just to blend suitably the quantity which is a grade which can acquire the effect and problems, such as superfluous ingestion, do not produce, and the quantity the ingestion which is 50 mg - about 1000mg/day is expected to be, if it is a case where the physiology effect of PS is solicited

[0026]As for phospholipid of this invention, it is preferred to use as an emulsifier and to add 0.01 to 10% to drugs, foodstuffs, cosmetics, etc. in that case.

---

[Translation done.]

EXAMPLE

---

[Example]Although an example is given to below and this invention is explained to it, this invention is not limited to these.

[0028]while taking 10.0 g of example 1 soybean lecithins (PC80: made by clo KURAN), and the soybean oil 2.0g into a 100-cc medium bottle, adding 90.0 g (100mL) ethyl acetate here and agitating with a stirrer -- warming -- it dissolved. **** picking and 0.1M sodium phosphate buffer solution (pH 7.0) 5.8mL was added, and L-serine 1.2g and PLD-Y1 (Made by Yakult Honsha) 1,500 unit were dissolved.

[0029]The medium bottle containing PC80 solution whole quantity is kept warm at 50 **, the whole quantity of the solution (L-serine+PLD-Y1) which kept it warm at 50 ** is added here, and a reaction is started, and it was made to react at 50 ** for 5 hours, stirring gently with a stirrer. After ice-cooling for 30 minutes, settling phospholipid and collecting, in boiling water, it was neglected for 20 minutes and the enzyme was deactivated.

[0030]Ethanol 40mL could be added to the collected phospholipid layer, and it mixed, and by neglecting it at 4 ** overnight, precipitate was made to form and supernatant liquid was collected. Ethanol 12mL could be further added to the precipitating part, it was neglected for 30 minutes after mixing, supernatant liquid was collected by centrifugal separation, and soybean transition lecithin / ethanol solution was obtained by mixing with previous supernatant liquid.

[0031]Thus, add 0.1M sodium acetate / ethanol solution to the obtained soybean transition lecithin / ethanol solution 2.0mL (about 0.33 g of solid content, PS content in phospholipid = 32.5%) 1.0 mL, and it is neglected at -20 ** for 1 hour, Centrifugal operation separated the formed precipitate (60 mg), and it washed by ethanol (PptNa-1). As a result of saving supernatant liquid for several days at -20 **, since the precipitate (7 mg) was produced further, centrifugal operation separated with supernatant liquid (SupNa, hardening-by-drying weight = 227 mg), and it washed by ethanol (PptNa-2).

[0032]After hardening each sample by drying, it dissolves in a diluent solvent (hexane: diethylether : isopropanol =2:2:1), After developing with thin layer chromatography (chloroform: developing solvent : methanol : acetic acid =13:5:2), under [ a fixed quantity / analysis system / gel pattern image / content / phospholipid / it makes phospholipid color with a Dittmer-Lester reagent and ]. A result is shown in the next table 1.

[0033]It was checked that, as for before fractionation, it turns out to having been 32.5% that it is [ which it is 81.9% in PptNa after fractionation ] 5.6% in SupNa, and PS can condense PS content (mol %) in phospholipid efficiently as shown in Table 1.
[0034]
[Table 1]

[0035]Reduced pressure drying of the soybean transition lecithin / the ethanol solution prepared in example 2 Example 1 was carried out, 900 mg of them was taken into the 50-cc medium bottle, and it was made to dissolve in the mixed liquor of chloroform 22.5mL and methanol 15mL. In this way, the prepared soybean transition lecithin / clo meta-solution are poured distributively into every 4.0 mL 6.0-cc medium bottle, 1M saline (a. a lithium chloride, b. potassium chloride, c. sodium chloride, d. magnesium chloride, e. calcium chloride, f. ammonium chloride, g. ammonium sulfate) was added here 0.8 mL. After shaking the bottle and mixing several times, it settled, chloroform layers were collected, and it took in the test tube which carried out weighing of the 1.0mL of them, and dried under nitrogen.
[0036]In this way, to the obtained dry matter (about 50 mg, a phospholipid content = about 25 mg), added 0.10-ml diethylether and dissolved, and added the ethanol of 1.0mL here gradually, precipitation was made to form, the whole suspension was centrifuged, and supernatant liquid and precipitation were divided.
[0037]An ethanol extract applies 5micro of things L by which 5microL and precipitation dissolved the whole for the diluent in chloroform of 2.5mL 2.5 times to a laminated plate, Under [ a fixed quantity / analysis system / gel pattern image / content / phospholipid / it makes phospholipid color after deployment and with a Dittmer-Lester reagent on condition of Example 1 and ]. A result is shown in the next table 2.

[0038]As shown in Table 2, the lithium chloride was the highest-achieving of eight kinds of the used salts, and PS preparation of 80% of purity was able to be obtained by the ethanol precipitation which is 1 time. It turned out that fractionation of about 3/4 of all the PA is carried out to ethanol supernatant liquid, and the removal efficiency of PA may be conspicuous for a lithium chloride.

[0039]

[Table 2]

[0040]

[Table 3]

[0041]The removal efficiency of PA was so good that the atomic number of the positive ion was small as an overall trend, and it turned out in the divalent positive ion that PC also precipitates. Phospholipid of most which contains PS in the case of ammonium salt did not precipitate, and it was not suitable for separation. The phospholipid content in ethanol precipitation and a supernatant liquid fraction is shown

in Table 3 about the high-achieving lithium chloride of precipitate, potassium chloride, and sodium chloride.

[0042]Reduced pressure drying of the soybean transition lecithin / the ethanol solution prepared in example 3 Example 1 was carried out, 900 mg of them was taken into the 50-cc medium bottle, and it was made to dissolve in the mixed liquor of chloroform 22.5mL and methanol 15mL. In this way, 1M lithium-chloride solution was added to the prepared soybean transition lecithin / chloroform methanol solution 8.0 mL, after shaking the bottle and mixing several times, it settled, and chloroform layers were collected and decompression hardening by drying was carried out.

[0043]Diethylether of 5.0mL was added to the obtained dry matter, and it dissolved in it, and the ethanol of 40mL was added here gradually, it dissolved (20 **), the whole suspension was centrifuged, and supernatant liquid (S1) and precipitation (P1) were separated. Diethylether of 5.0mL was again added to P1, and it dissolved in it, and the ethanol of 50mL was added here gradually, it dissolved (20 **), the whole suspension was centrifuged, and supernatant liquid (S2) and precipitation (P2) were divided.

[0044]That to which 5microL and S1 diluted with chloroform what soybean transition lecithin dissolved in 10 mg/mL, and dissolved P2 (damp or wet condition) in chloroform of 20 mg/mL 2.5 times 5microL, And apply undiluted solution 10muL to a laminated plate, phospholipid is made to color after deployment and with a Dittmer-Lester reagent on condition of Example 1, and S2 is the bottom about a fixed quantity of phospholipid contents by a gel pattern image analysis system.

[0045]PS content in P2 fraction which washed and obtained the ethanol precipitation thing by ethanol once again was 96.9%. Although the recovery rate in the ionic exchange method using CM cellulose was about (refer to reference example) 20%, the recovery rate in the fractionation method by this invention can serve as a useful refining means for about 90%, the effect evaluation it is high and using the high grade article, a break through of the mechanism of action, or drugs development.

[0046]

[Table 4]

[0047]Reduced pressure drying of the soybean transition lecithin / the ethanol solution prepared in example 4 Example 1 was carried out, 900 mg of them was taken into the 50-cc medium bottle, and it was made to dissolve in the mixed liquor of chloroform 22.5mL and methanol 15mL. In this way, the prepared soybean transition lecithin / chloroform methanol solution were poured distributively into every 4.0 mL 6.0-cc medium bottle, and 2M salt solution which dissolved in the citrate-sodium-acid-citrate buffer solution (pH 2.8, 3.6, 4.1, 4.6, 5.0) of various pH was added here 0.8 mL. After shaking the bottle and mixing several times, it settled, chloroform layers were collected, and it took in the test tube which carried out weighing of the 2.5mL of them, and dried under nitrogen.

[0048]To the dry matter (about 120 mg), diethylether of 0.2mL was added and it dissolved, and the ethanol of 2.0mL was added here gradually, it dissolved, the whole suspension was centrifuged, and supernatant liquid and precipitation were separated. An ethanol extract that by which 10microL and precipitation dissolved the whole for the diluent in chloroform of 10.0mL 10 times 5microL, Under [ a fixed quantity / analysis system / gel pattern image / content / phospholipid / apply to a laminated plate, and it makes phospholipid color with an after-deployment Dittmer-Lester reagent on condition of Example 1 and ].

[0049]On pH 3.6 or more conditions, both PS and PA were collected by the precipitation fraction (PA/PS=0.23), and were not able to separate both. On the other hand, a possibility that it could carry out fractionation of the PS in the state of sodium salt low (PA/PS=0.15) relatively if the amount of PA contained in precipitation repeats the ethanol treatment in this condition was shown by pH 2.8.

[0050]The result of having scoured the serine solution 190g (serine 70g+ water 120g)

and the solution (24 mg/mL) of phospholipase D (PLD-Y1, Yakult Honsha Make) to the soybean lecithin 200g of 40% of the example 5PC content 10 mL, and having made them reacting to it at 55 ** for 5 hours, The resultant whose PS content in phospholipid is 46.7% was acquired.

[0051]Ethyl alcohol 20mL was added to the resultant 5.0g, and ethyl alcohol 5mL extracted residue (precipitate) twice further after extraction at 45 **. 3 times of extracts are mixed, salt solution 0.20mL is added to 5mL of them 25%, it was neglected to the room temperature and precipitate was made to form after warming at 45 **. As a result, it turned out that it is 62.1% in a sediment and PS is efficiently condensed by the precipitating part to PS content in supernatant liquid having been 3.3% among the dry solid.

[0052]50 mg of sodium acetate powder is added to mixture 5mL of the extract prepared in example 6 Example 5, it was neglected to the room temperature and precipitate was made to form after warming at 45 **. As a result, it turned out that PS is efficiently condensed by the precipitating part as well as the case where are 61.8% in a sediment and a salt solution is used to PS content in supernatant liquid having been 3.5% among the dry solid.

[0053]Added the salt solution 25% to the mixture of an extract prepared in example 7 Example 5, the insoluble matter of PS was made to form, and PS content in precipitate phospholipid and the amount of PS collected by the precipitating part were measured. As the result, the relation between a salt addition, PS recovery rate to precipitate, and PS content in phospholipid is shown in Table 5.

[0054]

[Table 5]

[0055]PS was condensed by the precipitate fraction also on condition of any as shown in Table 5, but the quantity of the salt especially added to per [ phospholipid 1g in an extract mixture ] of PS content in precipitate phospholipid is not less than 55% in the range of 10 or less millimols, and PS was efficiently condensed by the precipitating part. On the other hand, when a salt addition was 0.05 or less millimols, the recovery rate of PS to precipitate is 60% or less, and not less than 40% existed in the supernatant liquid part. Although it was possible to have condensed PS from the above result to precipitate with any addition, the addition of the salt to the phospholipid 1g which dissolved in alcohol was considered to be the addition to which especially the range of 0.15 to 10 millimol was suitable for practical use.

[0056]Reference example It tried to refine PS from the soybean transition lecithin obtained in Example 1 according to the example of refining of PS from the refining bovine brains by CM cellulose column chromatography (the new chemical experiment lecture 4, lipid II phospholipid, p.127).

[0057](1) Preparation of Na+ type CM cellulose : suction **** was carried out, after adding CM-52 (Whatmann, swollen type) 50g gradually and settling it for about 30 minutes into 0.5N sodium hydroxide 500mL. It washed again after distilled water washed until **** became neutrality (500mLx2 time) until it passed 0.5N chloride 500mL and **** became neutrality with distilled water (500mLx2 time). After settling for 30 minutes in addition, stirring this gel in 0.5N sodium hydroxide 500mL, it washed

until it became neutrality with distilled water (500mLx1 time), and saved as methanol suspension eventually after washing by methanol 500mL further at the room temperature.

[0058](2) A column 30 mm in inside diameter is filled up with CM-52 adjusted by chromatography: (1) (bed volume 70mL). After having dissolved the soybean transition lecithin 1.0g of Example 1 in chloroform of 10mL after 500-mL-pouring chloroform and carrying out a conditioning, and applying the solution except precipitation to a column, you made it eluted under chloroform and the chloroform eluate fraction (Fr.1) of 200mL was obtained.

[0059]Chloroform methanol (85:15, v/v) 400mL (Fr.2+3), Chloroform methanol (75:25, v/v) 400mL (Fr.4+5), Chloroform methanol (65:35, v/v) 400mL (Fr.6+7) and chloroform methanol (50:50, v/v) 400mL (Fr.8+9) were passed, fractionation of every 200 mL was carried out, and silica gel thin layer chromatography analyzed phospholipid.

[0060]As a result, although phospholipid was hardly detected by the chloroform eluate fraction (Fr.1), 0.29g (dry weight) elution of the thing of the almost same presentation as the ethanol precipitation thing applied by the cloudy state was carried out in the first half (Fr.2) of a chloroform methanol (85:15, v/v) fraction. Although PC was not contained in the chloroform methanol (75:25, v/v) fraction (Fr.4+5), both PS and PA are contained, and it was mostly eluted especially in PA. And purity was [ that PA is not contained on and after the second half (Fr.7), but coloring slight at the starting point in addition to PS is only seen, and ] 97.7% although 8.0% of PA was still contained in the first half (Fr.6) of a chloroform methanol (65:35, v/v) fraction. Although a considerable amount of PS was contained in the first half (Fr.8) of a chloroform methanol (50:50, v/v) fraction, there was almost no effluent in the second half (Fr.9).

[0061]Although it became clear from the above result that PS can be refined also from soybean transition lecithin by the cation-exchange chromatography of the almost same conditions as a bovine brain fraction, It turned out that it is the method of not being suitable for a recovery rate obtaining a lot of refining PS very low (it is about 20% when Fr.7 or subsequent ones are collected).

[0062]

[Translation done.]